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Fluconazole Susceptibility Testing of *Candida albicans*: Microtiter Method That Is Independent of Inoculum Size, Temperature, and Time of Reading

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In vitro antifungal susceptibility testing generally remains unstandardized and unreliable for directing therapy. When azoles are tested, this problem is further compounded by the lack of definite reading end points. We determined the in vitro susceptibility of 50 *Candida albicans* isolates (including 10 reference strains) to fluconazole by using a microbroth dilution method in which microtiter plates were agitated immediately before reading. Six fungal inoculum sizes (ranging from 2×10^2 to 4×10^5 CFU/ml), three different times of reading (24, 48, and 72 h), and two temperatures (30 and 35°C) were tested. We also compared visual and spectrophotometric determinations of MIC end points. This agitation method resulted in clear-cut visual end points that were reproducible for different observers within the same laboratory, that were independent of inoculum size, temperature of incubation, and time of reading, and that correlated well with the degree of fungal inhibition as determined by spectrophotometry. Median MICs also correlated with usually achievable levels of fluconazole in serum and tissue of humans and experimental animals.

Over the past several years, we have witnessed an increasing number of opportunistic fungal infections in immunocompromised patients (1, 5, 14, 28). In addition, fungi previously considered harmless colonizers have now emerged as significant pathogens, and many of them are resistant to available drugs (4, 19, 24). At the same time, several new antifungal agents have become available, resulting in a great demand for in vitro susceptibility testing (9, 13, 20, 23, 25, 26). Although major advances in antifungal susceptibility testing have been achieved, particularly through the efforts of the National Committee for Clinical Laboratory Standards (NCCLS) (2, 6, 8–10, 21, 22), currently available methods remain difficult to duplicate and cannot be relied upon for directing therapy (6, 9, 10, 26). This problem is particularly relevant to tests of susceptibility to azoles because of the lack of definite reading end points due to the "trailing effect." In this report, we present results of our determination of the in vitro susceptibility of 50 *Candida albicans* isolates to fluconazole by using a microbroth dilution method with agitation of the plates immediately before reading. This method resulted in clear-cut visual end points that were reproducible; that were independent of inoculum size, time of reading, and incubation temperature; and that correlated with the degree of fungal inhibition as determined by turbidity studies.

MATERIALS AND METHODS

Antifungal agent. Fluconazole (Pfizer, Sandwich, United Kingdom) was rendered soluble in 100% dimethyl sulfoxide (Sigma Chemical Co., St. Louis, Mo.) at a starting concentration of 6,400 μg/ml. Fluconazole was diluted with assay medium to the highest drug concentration of 64 μg/ml (4% dimethyl sulfoxide). Twofold dilutions were made for the remaining wells according to the NCCLS's modification of Ericsson and Sherris' method of dilution (8).

Assay medium. RPMI 1640 medium powder with L-glutamine and without phosphate buffer (Sigma Chemical Co.) was used as the assay medium. Preweighed aliquots of 10.4 g of medium were mixed with 1,000 ml of deionized water buffered with morpholinepropanesulfonic acid (MOPS; Sigma Chemical Co.). The buffer solution was prepared with 34.53 g of MOPS (0.165 M) per 1,000 ml of water. Clear solutions of both the buffer powder-water and medium powder-buffered water combinations were obtained by using magnetic spin bars at room temperature. The solutions were titrated by using 10 N sodium hydroxide to pH 7, filter sterilized by using 0.22-nm filters, aliquoted into 50-ml Falcon tubes, and stored at 4°C. Sterility checks were performed by inoculating 1 ml of solution to a blood agar plate, which was then incubated at 35°C for 7 days and read daily.

Microtiter plates. We employed sterile, individually wrapped plastic assay plates containing 96 round-bottom wells each (Dynatech Laboratories, Inc., Alexandria, Va.). The stock solutions of fluconazole were dispensed in assay medium to obtain the appropriate concentrations in wells 1 through 10 in each row; drug-free medium was dispensed in wells 11 and 12. Well 11 served as growth control, and well 12 served as a sterility check as well as a blank control for the spectrophotometric assay. The drug concentration ranged from 64 to 0.125 μg/ml, obtained by 10 twofold serial dilutions. A representative microtiter plate was incubated at 35°C for 3 days and read daily to ensure sterility. The plates were wrapped in aluminum foil to minimize dehydration during storage in the −70°C freezer. Thawing was accomplished by setting the plates in the 4°C refrigerator for 1 h, leaving them at room temperature for another 1 h, and finally placing them in the 35°C incubator for 15 to 30 min to minimize condensation formation on the plate lids. The plate lids also were propped up slightly to allow evaporation of condensate during all three stages of the thawing procedure.

Inoculum. Starting inocula of *Candida albicans* were obtained by picking five colonies of >1 mm in diameter from 24-h-old cultures. The yeast cells were suspended in 5 ml of sterile 0.85% saline. The resulting yeast suspension was

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vortexed for 15 s, and the turbidity was adjusted to 1×10^6 to 5×10^6 CFU/ml by using spectrophotometric methods as recommended by the NCCLS (21) (530 nm, 85% transmittance). Dilutions of 1:3, 1:10, 1:20, 1:200, and 1:2,000 were used in the experiments assessing the role of inoculum size. Colony counts were performed to confirm the number of CFU per milliliter for each of the inoculum sizes tested. The inoculum concentration ranged from 5×10^6 to 2×10^3 CFU/ml.

Microtiter plates were inoculated by using an automatic pipettor (Rainin Instrument Company, Woburn, Mass.) programmed to pick up 140 μl of inoculum suspension and to dispense 10 μl in each of the wells. The inoculum sequence proceeded from well 11 to well 1 (from the well containing the lowest drug concentration to that containing the highest) by using a single set of pipette tips for each plate. All organisms were tested in duplicates in each run of the experiments.

Incubation time and temperatures. Inoculated plates were incubated at either 30 or 35°C. The incubation time was 24, 48, or 72 h. Daily readings were made visually and spectrophotometrically before and after agitation of the plates. Agitation was accomplished with a microtiter plate shaker (two-plate model; Dynatech Laboratories, Inc.) set at 50 calibrations for 5 min. This calibration was used to allow maximum agitation leading to the formation of a homogenous suspension in the growth control well. Spectrophotometric readings were performed with a Dynatech automatic plate reader (model MR600) set at single beam, 199 calibrations, 199 threshold at 570 nm. In addition to visual and spectrophotometric readings, four randomly selected organism cultures were also examined for growth inhibition by subculturing 10 μl from each of the 12 wells on Sabouraud dextrose agar plates after adequate dilution.

End point criteria. Two different sets of parameters were used to grade the growth in the wells. The following classifications were used for readings before agitation: 4+, >75 to 100% growth; 3+, >50 to 75% growth; 2+, >25 to 50% growth; 1+, >0 to 25% growth; and 0, no growth. Control wells were assigned 100% growth (4+), and growth in the other wells was compared with that in the control well. The MIC end point was defined as the point at which growth was less than or equal to 1+. Similar end point criteria have been previously used by the NCCLS on antifungal susceptibility testing (22).

The MIC end point after agitation was defined as the first drug concentration well exhibiting approximately 50% (or more) reduction of growth compared with the control well. The relative inhibition of growth compared with the control was determined by spectrophotometry, and the MIC end point by turbidity was defined as the first drug concentration at which turbidity in the well was more than 50% less than that in the control well.

Organisms. Fifty *C. albicans* strains were tested. Ten of these were American Type Culture Collection strains (no. 64544 through 64553). Three resistant strains (R89-208, R89-214, and R89-335) were kindly provided by the Fungus Testing Laboratory (M. Rinaldi, San Antonio, Tex.). An additional resistant strain, *C. albicans* KB, was also tested (kindly provided by C. Kirkpatrick, Denver, Colo.). The remaining strains were isolated from the blood of patients with cancer cared for at The University of Texas M.D. Anderson Cancer Center. Isolates were maintained in water stocks; they were subsequently subcultured to chocolate agar plates to ensure purity and further subcultured to Sabouraud dextrose agar plates. Twenty-four-hour growth was used in all experiments. Repeat experiments were performed with organisms from 24-h subcultures of the original Sabouraud dextrose agar plates whose *Candida* organisms had been obtained from the water stocks. Water stocks were maintained at room temperature, and all subcultures were incubated at 35°C for 24 h and then kept at room temperature for reference or used for further subcultures when needed. The organisms were coded by a technologist not involved in the study, and the code was not broken until completion of all the experiments.

TABLE 1. Effect of agitation on fluconazole MICs for 50 *C. albicans* isolates

MIC (μg/ml)	No. of strains with:	
	No agitation	Agitation
0.125	0	14
0.25	0	25
0.50	0	6
8.0	0	1
16.0	1	1
32.0	1	0
64.0	9	2
>64.0	39	1

RESULTS

The agitation of the microtiter plates yielded clear-cut MIC end points. MICs of fluconazole obtained by direct visual reading without agitation, however, were difficult to read and were in the resistant range (MIC for 50% of the isolates tested [MIC_{50}], >64 μg/ml; range, 16 to >64 μg/ml) (Table 1), not in keeping with the known activity of this drug in experimental and human candidiasis (7, 13, 17, 18, 20). Before agitation, the visible growth for the susceptible strains was noted at 64 μg/ml, gradually increasing as the drug concentration fell to 0.125 μg/ml but without any significant differences between consecutive wells, which would have allowed end point determination (Fig. 1). Cultures of the resistant strains showed significant turbidity in all wells, with some progressive reduction in turbidity at higher fluconazole concentrations. Cultures with apparent growth near or equivalent to that in the control wells that rated 3+ or 4+ before agitation appeared to have <50% growth by either visual observation after agitation or spectrophotometry (Fig. 1).

Reproducible results were obtained only after agitation of the plates. Of the 50 strains tested, most isolates appeared susceptible (MIC for 90% of the isolates [MIC_{90}], 0.50 μg/ml) compared with the MIC_{50} of >64 μg/ml prior to agitation (Table 1). Blind repeat testing (≥3 times) of the same organisms yielded MIC end points that were always within one twofold dilution even when read by three different observers in the same laboratory (data not shown). The results obtained after agitation were independent of inoculum size despite the wide range of inoculum sizes tested (from 2×10^2 to 5×10^5 CFU/ml) (Table 2). Results were also independent of time of reading and temperature of incubation: the MIC remained unchanged at 0.25 μg/ml (range, 0.125 to >64 μg/ml) whether the plates were read at 24, 48, or 72 h (Table 3); similarly, the temperature of incubation (30 versus 35°C) did not have any effect on the observed values (mean, 0.25; range, 0.125 to >64 μg/ml) (Table 4). Results of tests assessing time of reading or

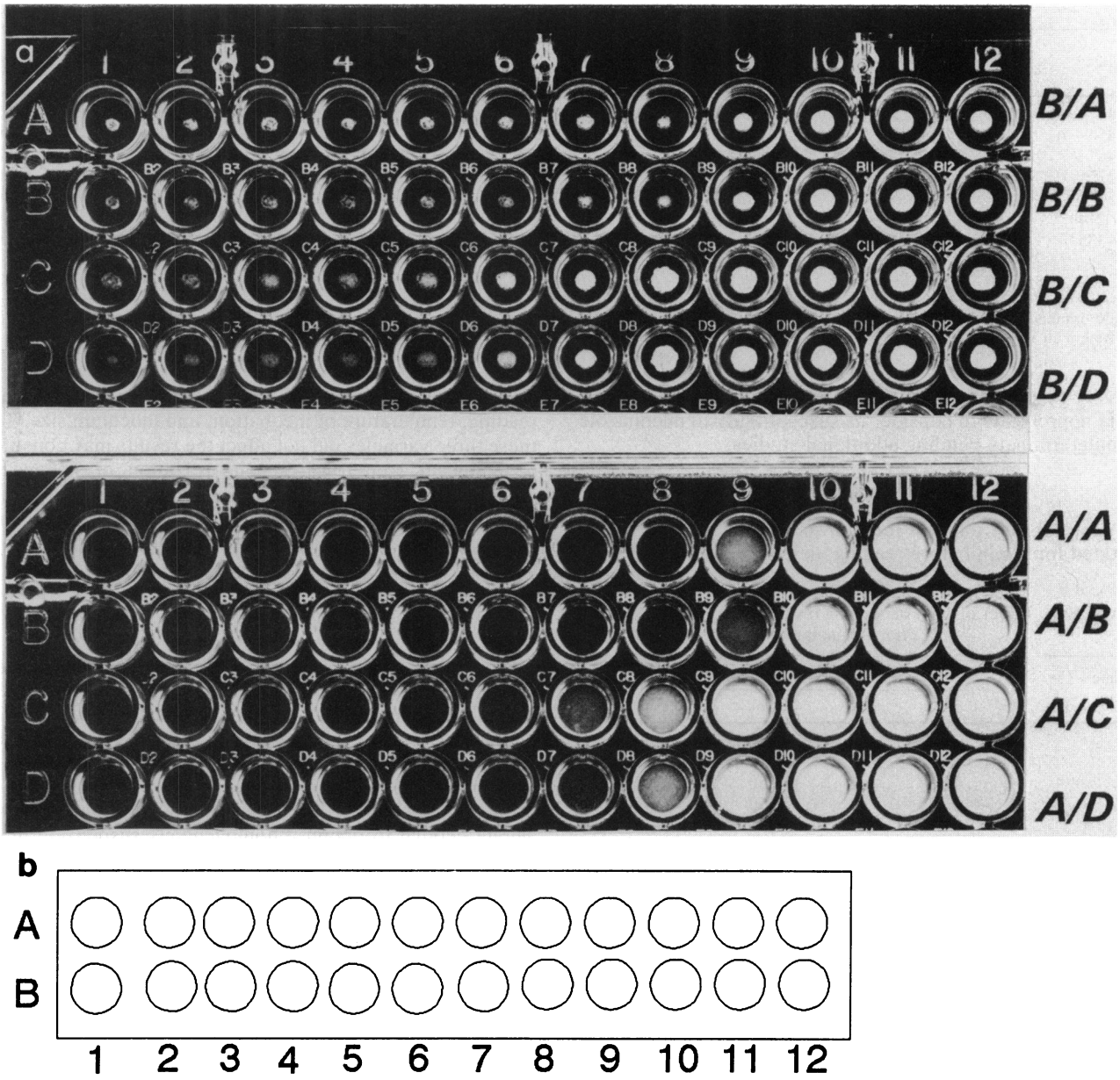

FIG. 1. (a) Growth pattern of two strains of *C. albicans* tested in duplicate in the presence of increasing fluconazole concentrations. B/A to B/D, trailing seen before agitation; A/A to A/D, clearer end points after agitation. (b) A and B are duplicate determinations. Wells 12 are growth control wells; wells 11 to 1 contain increasing fluconazole concentrations (0.0625 to 64 μg/ml).

incubation temperature yielded MICs that were always within one twofold dilution difference.

End points determined by visual reading after agitation correlated well with those obtained by spectrophotometric assay (Table 5) and with those obtained by counting CFUs when such counting was done (data not shown). Of 38 isolates in blind tests with both visual and spectrophotometric reading, 31 had exactly the same MICs, and only a twofold (one well) difference was obtained for the remaining seven: higher MICs by visual reading for four isolates and lower readings for three. The MIC_{90}s and ranges determined for both visual and spectrophotometric methods were identical (MIC_{90}, 0.50 μg/ml; range, 0.125 to 16 μg/ml) (Table 5).

The degree of inhibition of the susceptible strains determined by spectrophotometry ranged from 55 to 99%, with a median of 75% and a mean of 76%. Similar degrees of inhibition were seen with the resistant strains but at much higher drug concentrations, reflecting their higher MICs.

Tests with strain R89-208 gave results that were difficult to interpret. No clear cutoff point could be readily discerned for this organism, although on second inspection we found that cultures containing 0.5 ml of fluconazole had a significantly reduced amount of turbidity after agitation. Unlike the susceptible strains, which showed a median 75% reduction of growth by spectrophotometry, R89-208's growth was inhibited by an average of 45 to 50% but never more than 50%.

On the basis of these findings, it may be more difficult to

TABLE 2. Effect of inoculum size on fluconazole MICs for nine *C. albicans* isolates

Isolate	MIC[a] (μg/ml) per inoculum size (CFU/ml) of:					
	5×10^5	1×10^5	5×10^4	1×10^4	2×10^3	2×10^2
64544[b]	0.250	0.250	0.250	0.250	0.500	0.250
64545[b]	0.500	0.500	0.500	0.500	0.500	1.000
64546[b]	0.500	0.500	0.250	0.250	0.250	0.250
64547[b]	0.250	0.250	0.250	0.250	0.125	0.250
64550[b]	16.000	16.000	16.000	16.000	16.000	16.000
R89-214	>64.000	32.000	>64.000	>64.000	32.000	16.000
R89-335	64.000	64.000	64.000	64.000	64.000	32.000
602	0.250	0.250	0.250	0.250	0.125	0.125
604	0.250	0.250	0.250	0.250	0.250	0.250

[a] Determined visually after agitation.
[b] American Type Culture Collection strain.

classify this organism as susceptible or resistant. It may be more appropriate to consider its susceptibility to fluconazole as indeterminate pending additional studies.

DISCUSSION

A microbroth dilution method in which the plates were agitated for 5 min before reading yielded definite and reproducible MIC end points that were independent of time of reading, temperature of incubation, and inoculum size. That these three variables did not affect the results may constitute a significant step toward standardization of fluconazole susceptibility testing. The agitation did not affect the actual number of organisms in the well but rather their distribution in it. After a 24-h incubation period, the organisms formed a pellet which was easily visualized through the MIC reading mirror at the bottom of the well. However, the thickness (and therefore the volume) of the pellet, which may differentiate between the wells containing larger numbers of organisms (resistant) and those with much lighter growth (susceptible), could not be appreciated. With vigorous agitation, the sediment dispersed and formed different degrees of turbid suspensions depending upon the number of organisms present in the wells. Hence, this method may allow an indirect quantitation of growth. The calibration criterion for the agitation method is not important. What is important is to agitate enough to obtain a homogeneous suspension in the growth control well.

TABLE 3. Effect of time of reading on fluconazole MICs for 36 *C. albicans* isolates

Isolate no.	MIC[a] (μg/ml) at the following time:		
	24 h	48 h	72 h
604	0.250	0.250	NT
622	0.250	0.250	0.250
623	0.250	0.250	0.250
624	0.125	0.250	0.250
625	0.125	0.250	0.250
626	0.250	0.250	0.250
627	0.125	0.250	0.250
628	0.125	0.250	0.250
632	0.125	0.250	0.125
633	0.125	0.250	0.125
634	0.250	0.250	0.250
635	0.125	0.125	0.125
636	0.250	0.250	0.250
638	0.250	0.250	0.250
639	0.125	0.125	0.125
640	0.250	0.250	0.250
641	0.250	0.250	0.250
642	0.125	0.250	0.125
643	0.125	0.125	0.125
644	0.250	0.250	0.250
645	0.125	0.250	0.250
646	0.250	0.250	0.250
647	0.250	0.250	0.250
648	0.250	0.250	0.250
649	0.250	0.250	0.250
650	0.250	0.250	0.250
64544[b]	0.500	0.250	NT
64545[b]	0.500	0.500	NT
64546[b]	0.500	0.500	NT
64547[b]	0.250	0.250	0.250
64548[b]	0.500	0.500	NT
64549[b]	0.250	0.250	NT
64550[b]	16.000	16.000	16.000
64551[b]	0.125	0.125	NT
64552[b]	0.125	0.125	NT
64553[b]	0.500	0.500	NT

[a] Incubation temperature, 35°C. NT, not tested.
[b] American Type Culture Collection strain.

The microbroth used in this method is simple to prepare, has a long shelf life when kept at −70°C, and requires only the addition of the fungal inoculum. It is inexpensive and can be easily used in routine clinical microbiology laboratories.

The end points determined by both visual and spectrophotometric readings suggest that the latter method could be used as an alternative to visual reading for the rapid screening of antifungal compounds since it easily lends itself to automation.

Of note, the fluconazole MIC end points obtained for susceptible organisms (0.25 μg/ml) by using this method correspond to achievable levels of fluconazole in blood and tissue of humans and experimental animals and are in agreement with the known in vivo activity of the drug in the setting of human and experimental candidiasis (7, 17, 18, 20, 25). Additional experiments are currently ongoing to correlate our in vitro findings with in vivo results of treatment in a murine model of systemic candidiasis.

Despite the interesting findings observed with this method, we emphasize that fluconazole was the only drug tested and that MICs for one organism (R89-208) were not as clear-cut as those obtained for the remaining 49 organisms. However, preliminary results indicate that this method may also be valid for other fungi and other drugs, including ketoconazole, SCH 39304, and 5-flucytosine (data not shown). Although this method yielded reproducible results among different observers in the same laboratory, its reproducibility between laboratories has not been tested.

TABLE 4. Effect of temperature of incubation on fluconazole MICs for 20 *C. albicans* isolates

Isolate no.	MIC[a] (μg/ml) at the following temperature:	
	35°C	30°C
626	0.250	0.250
627	0.250	0.250
634	0.250	0.250
635	0.250	0.250
646	0.250	0.250
647	0.250	0.250
648	0.250	0.250
649	0.250	0.250
KB	8.000	8.000
64544[b]	0.250	0.250
64545[b]	0.250	0.500
64546[b]	0.250	0.250
64547[b]	0.250	0.250
64548[b]	0.250	0.250
64549[b]	0.250	0.250
64550[b]	16.000	16.000
64551[b]	0.250	0.250
64552[b]	0.125	0.125
64553[b]	0.250	0.250
R89-208	>64.000	>64.000

[a] Incubation time, 24 h.
[b] American Type Culture Collection strain.

Results of correlations between our visual and turbidimetric readings and colony counts in a limited set of experiments agreed with those of others (15), showing good correlation among all three methods.

We caution the reader, however, that the MICs obtained in in vitro tests on susceptible or resistant strains do not represent clinically useful cutoff points for in vivo susceptibility to fluconazole. Rather, these cutoff points will have to be derived from prospective clinical studies in humans. It is also important to keep the results of in vitro susceptibility testing in perspective. Other factors, such as pharmacokinetics, interaction with endogenous enzyme systems, and particularly the immune status of the host, play essential roles in the final outcome of fungal infections.

Galgiani and Stevens (11, 12) described an in vitro susceptibility testing method based on turbidimetric evaluation of growth and determination of the drug concentration that produces 50% inhibition compared with the drugfree control ($IC_{1/2}$). Their test was found to be inoculum independent and constituted a significant advance in antifungal susceptibility testing. Furthermore, when modifications to this test were applied (IC_{50} and IC_{30}) (12, 16), ketoconazole-resistant *C. albicans* isolates could for the first time be separated from susceptible ones. Our agitation method probably measures end points similar to those measured by the $IC_{1/2}$, the IC_{50}, or the IC_{30}. Of interest are the similarities between MICs of fluconazole for strains of *C. albicans* obtained with our method (0.25 μg/ml) and those obtained by McIntyre by using the method developed by Galgiani et al. (0.16 μg/ml) (20). However, the agitation method does not require the availability of a spectrophotometer or additional mathematical calculation for end point determination. Also, our method does not necessitate additional dilution of cultures and graphic determination of end points or special equipment (12, 16). The only necessary equipment is a plate shaker (2-Plate Minishaker; Dynatech Laboratories, Inc.). Hence, this method could be easily used in standard clinical laboratories.

TABLE 5. Comparative evaluation of visually and spectrophotometrically determined MICs for 30 *C. albicans* isolates[a]

MIC (μg/ml)	No. of strains		No. of exact matches[b]
	Visual	Spectrophotometric	
0.125	14	13	12
0.250	18	22	17
0.500	5	1	1
1.000	0	1	0
16.000	1	1	1

[a] Strains were incubated at 35°C for 24 h.
[b] All numbers of strains per MIC were within twofold of each other.

Gordon et al. (13) recently described a semisolid agar method for susceptibility testing of azoles that reportedly produced sharp end points. This method is time-consuming and may not be very practical for use in a clinical laboratory but seems well suited for a research laboratory. In addition, we have not found that it eliminates the trailing effect that occurs when testing the susceptibility of strains of *C. albicans* to azoles (3, 27). It may well be that subtle changes in methodology yielded significant differences between our results by the semisolid agar method and those previously reported by Gordon et al. Additional studies may help to clarify this issue.

In conclusion, a microbroth dilution method employing prereading agitation yielded definite MIC cutoff points when the in vitro susceptibility of *C. albicans* to fluconazole was tested. Results obtained by using this method were identical by visual and spectrophotometric reading and were independent of inoculum size, time of reading, and temperature of incubation. This method is inexpensive, flexible, and easy to perform in standard clinical laboratories may be applicable to most other antifungal drugs. A multi-institutional comparative evaluation of this method, which is being conducted in collaboration with the NCCLS, and extensive in vivo correlation, both in humans and in animals, are currently ongoing to determine the method's interlaboratory reproducibility and its potential usefulness as a guide to antifungal therapy.

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